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(54) Bezeichnung: EIN EXPRESSIONSSYSTEM FÜR DIE ANAEROBE GENEXPRESSION IN HÖHEREN PFLANZEN

(57) Abstract

The invention relates to an expression system for anaerobic gene expression in higher plants. The expression system according to the invention comprises the promoter GapC4 or parts or variants thereof and a gene to be expressed.

(57) Zusammenfassung

Die Erfindung betrifft ein Expressionssystem für die anaerobe Genexpression in höheren Pflanzen. Das erfindungsgemäße Expressionssystem umfaßt den Promoter GapC4 oder Teile oder Varianten des Promotors GapC4 und ein zu exprimierendes Gen.

An expression system for anaerobic gene expression in higher plants

The present invention relates to an expression system for anaerobic gene expression in higher plants. A concrete field of application of the present invention is agriculture, particularly resistance cultivation and the increase in the efficiency of useful plants.

The loss of harvested crops, which results from diseases of plants, represents a world-wide problem. For example, the disease of potatoes suffering from potato wet rot or potato rot (rotting of the tuber) and black leg or black stem (rotting of the lower stem sections) after infection by the phytopathogenic bacterium *Erwinia carotovora*, results in crop losses to an estimated amount of 100 million dollars world-wide (Pérombelon and Kelman, 1980, Ann. Rev. Phytopathol., 18, 361-387). There is a number of studies dealing with the transmission of resistance factors to plants by means of genetic engineering (Lamb et al., 1992, Bio/Technology, 10, 1436-1445; Hain and Fischer, 1994, Current Opinion in Biotechnology, 125-130; Zhu et al., 1994, Bio/Technology, 12, 807-812). In order to increase the resistance of potatoes to *Erwinia carotovora,* the T4 lysozyme gene of the bacteriophage T4 was expressed in transgenic potatoes (Düring et al., 1993, Plant J. 3, 587-598).

However, since bacterial diseases of plants often spread under anaerobic conditions, the resistance factors for plants, transmitted so far, become effective only to a very restricted extent. This applies particularly to the above-mentioned disease of potatoes suffering from potato rot and black leg, since the infection caused by *Erwinia carotovora* takes place predominantly under anaerobic conditions. This effect is even increased by the formation of the mucus from bacteria and degradation products of vegetable cell membranes. Regarding an effective expression of an antibacterial protein under optimum conditions, the control of the corresponding foreign gene by a promoter active under these conditions has thus to be aimed at.

3 anaerobic promoters have been tested in transgenic plants so far: These are the *Adh1* promoter from corn, the *Adh* promoter from *Arabidopsis thaliana* and the *GapC* promoter from *Arabidopsis thaliana*. The *Adh1* promoter from corn was investigated in tobacco and rice (Ellis et al., 1987, EMBO J. 6, 11-16; Kyozuka et al., 1991, Mol. Gen. Genet. 228, 40-48), the *GapC* promoter from *Arabidopsis* was investigated in tobacco (Yang et al., 1993, Plant Physiol. 101, 209-210), and the *Adh* promoter from *Arabidopsis* was investigated in *Arabidopsis* as such (Dolferus et al., 1994, Plant Physiol. 105, 1075-1087). In this connection, it turned out that all promoters convey only 2 to 81 times the induction of the reporter gene over the background and are not active in all of the tissues.

In one aspect of the present invention there is provided a system for the expression of resistance factors in useful plants and resultant transgenic plants.

In a second aspect of the present invention there is provided the anaerobic expression of the T4 lysozyme gene in potatoes and corresponding transgenic plants. In a related aspect of the present invention there is provided potatoes resistant to phytopathogenic bacteria.

The invention is realised using an expression system comprising the GapC4 promoter or parts or variants of the GapC4 promoter operably linked to a gene to be expressed.

As used herein, parts or variants of the GapC4 promoter are intended to include those parts or variants of the nucleotide seq of the GapC4 promoter which have an inducibility under anaerobic conditions substantially comparable to that of the wildtype GapC4 promoter. Such parts or variants can be isolated by conventional methods, for example, by shortening the length of the wildtype promoter or substituting one or more alternative bases for those present in the wildtype GapC4 promoter, as will be appreciated by those of skill in the art to which this invention relates. Examples of such variants are provided herein under the

heading 'Example'. Similarly, those of general skill in the art will understand that parts and variants of the wildtype GapC4, which possess inducibility under anaerobic condition substantially comparable to that of the wildtype GapC4 promoter, may be identified utilising activity assays such as that described herein under the heading 'Example'.

According to the invention, genes for an antibacterial protein, particularly for T4 lysozyme, resistance genes (r-determinants) against viruses, nematodes, bacteria and fungi, genes having an insecticidal effect, glycolysis-increasing genes and fermentation-increasing genes are concerned.

According to the invention, the expression system is applied to the anaerobic gene expression in higher plants. It is used preferably in cultivated plants such as potatoes, rice, grain, corn, tomatoes, brassicaceae, leguminous plants, cotton, sugar beets and carrots.

This invention also relates to higher plants, preferably transgenic cultivated plants such as potatoes, rice, grain, corn, tomatoes, brassicaceae, leguminous plants, cotton, sugar beets and carrots, which contain the expression system according to the invention.

Transgenic potatoes which contain an expression system from GapC4 promoter and the gene for T4 lysozyme are of special importance.

The major advantage of the *GapC4* promoter used according to the invention (GenBank accession No. L40803) consists in that it has an induction profile excellently suited for the objective. The anaerobic expression achieves the intensity of the 35S promoter of cauliflower mosaic virus (35S CaMV) which is frequently used under aerobic conditions for the expression of foreign genes. Furthermore, the promoter is active in all tissues such as flower or blossom, leaf and root. The isolation of the *GapC4* gene and the anaerobic induction of the *GapC4* promoter in a transient expression system in suspension culture cells of corn have been published so far (Kersanach et al., 1994, Nature 367:387-389).

Surprisingly, the promoter is especially active in the cultivated plant of potato.

Further possible applications of the invention:
Another problem is represented, especially in moist climate, by periodic flooding of fields, which may lead to crop failure. As they are aerobic organisms, plants cannot survive prolonged periods of extensive moisture which results in a decrease of oxygen available to the plants (Perata and Alpi, 1993, Plant Sci. 93, 1-17). The tolerance of plants towards insufficient oxygen supply differs rather widely for individual species. For example, the embryo in rice caryopsis also germinates under these conditions without any difficulties, whereas corn germs survive without oxygen for only about 24 hours. A general adaptive strategy of higher plants to anaerobic conditions is the increase in glycolysis as well as the starting of fermentation processes. In order to increase the tolerance of plants towards insufficient oxygen supply, the genes which take part in the glycolysis

as well as the fermentation can be controlled by an anaerobically inducible promoter. These genes are then expressed in the case of insufficient oxygen supply.

The present invention is explained in more detail below by means of the examples.

Example

In order to investigate whether the *GapC4* promoter of potatoes is induced anaerobically, and induced after the infection with *Erwinia carotovora, GapC4* promoter-reporter gene constructs are transformed into the potatoes. Following the infection of the transgenic potatoes with *Erwinia carotovora* and the incubation of tissues of the transgenic potatoes under anaerobic conditions, the reporter gene expression can be measured. The reporter gene can then be replaced *in vitro* by the T4 lysozyme gene and transformed into the potatoes. Thereafter, the transgenic potatoes are investigated for increased resistance to *Erwinia carotovora*.

The reporter gene constructs were constructed as described below:
All agrobacterium T-DNA constructs are based on the binary vector pOCA28 (Honma et al., 1993, Proc. Natl. Acad. Sci., USA 90, 6242-6246; Olszewski et al., 1988, Nucleic Acids Res. 16, 10765-10782). Plasmids pUK443 and pUK444 carrying 785 base pairs of the *GapC4* promoter and 461 base pairs thereof, respectively, the first intron of the *GapC4* gene as well as the ß-glucuronidase reporter gene were used for T-DNA constructs. In order to exclude possible negative effects of the *GapC4* intron from corn in transgenic tobacco plants and potatoes, the intron was cut out of the plasmids pUK443 and pUK444 by restriction digestion using enzymes *XhoI* and *NcoI*. Following a anaplerotic reaction of the ends resulting from restriction digestion, the pUK403 and pUK404 plasmids without introns were produced. The promoter-reporter gene fragments of these plasmids were cut out by *PvuII* digestion and cloned

into the *SmaI* restriction site of pOCA28. The resulting pOCA28 derivatives, pUK4030, 4040 and 4041, carry the *GapC4* promoter-reporter gene construct. The ß-glucuronidase reporter gene without promoter was cloned into pOCA28 as a control. pUK4030 and 4040 carry the reporter gene constructs with the promoter being proximal relative to the right-hand T-DNA border sequence. In pUK4041, the reporter gene is oriented differently.

All of the recombinant DNA techniques are carried out according to standard protocols (Sambrook et al., 1989, Molecular cloning, A laboratory manual. New York: Cold Spring Harbor Laboratory Press).

For cloning the T4 lysozyme gene the ß-glucuronidase reporter gene can be removed from plasmids pUK403 or 404 by restriction digestion, and the T4 lysozyme gene can be cloned thereinto instead. The T4 lysozyme gene controlled by the *GapC4* promoter is recloned into an agrobacterium T-DNA vector. Following the introduction into *Agrobacterium tumefaciens*, all constructs are transformed into the potatoes according to standard protocols (Düring et al., 1993, Plant J. 3, 587-598; Fladung 1990, Plant Breeding 104, 295-304). Transgenic potatoes are selected by means of an antibiotic resistance gene on the T-DNA and investigated for expression of the introduced ß-glucuronidase reporter gene and the T4 lysozyme gene under anaerobic conditions as well as after infection with *Erwinia carotovora*.

For the purpose of anaerobic induction, plant tissue is incubated in an air-tight glass container (Merck) together with Anaerocult A (Merck) for at least 12 hours. For the fluorimetric GUS assay, the plant material is homogenized and incubated with the ß-glucuronidase substrate 4-methylumbelliferyl-ß-D-glucuronide (MUG) at 37°C. Fluorescence quantification is carried out according to Jefferson et al., EMBO J. 6, 3901-3907 (1987) and protein concentrations are determined according to Bradford, Anal. Biochem. 7, 248-254 (1976). In order to measure the tissue specificity of the reporter gene expression, the intact anaerobically induced plant material is infiltrated *in vacuo* with a solution of 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid) and incubated at 37°C overnight. For making the staining or coloring better visible, the chloro-

phyll is extracted using 70 % of ethanol (Jefferson, see above).

In order to measure the expression of the T4 lysozyme gene, Northern blot analyses are carried out using a T4 lysozyme-specific probe after anaerobic incubation of the tissue. Whole RNA is isolated by means of the RNeasy kit (Qiagen). The concentration of the RNA is determined photometrically and 10 micrograms of the RNA are placed in a lane on a 1 % agarose gel containing formaldehyde. Following electrophoresis, the RNA is blotted from the gel onto nitrocellulose or nylon membranes (Amersham) using 0.05 N NaOH as transfer buffer (Sambrook et al., see above). The T4 lysozyme-specific probe is marked and hybridized with the RNA filter according to standard conditions (Sambrook et al., see above, Düring et al., see above).

After confirmation of the anaerobic induction of reporter gene and T4 lysozyme gene, investigations are made as to whether both genes are induced even after the infection by the phytopathogenic bacterium *Erwinia carotovora* and whether the T4 lysozyme gene is activated under the control of the GapC4 promoter such that it conveys resistance, respectively. The induction of reporter gene and T4 lysozyme gene is determined as described above. For this purpose, the tuber or bulb material underneath the macerated tissue is utilized.

The infection test is carried out with a pathogenic strain of *Erwinia carotovora ssp. atroseptica* or *ssp. carotovora* in plastic containers in the absence of air. Slices having a defined size are produced from potato tubers and are inoculated in the middle with a defined number of bacterial cells in a freshly cut state in a small volume. Incubation takes place in plastic containers having a water layer on the bottom on a soaked filter paper. As a result, saturated humidity will be achieved. The bacterial growth is traced by means of tissue maceration and the resulting bacterial mucus. Because of the bacterial mucus that forms, potato cells are covered in an air-tight fashion. As a function of the inoculin density, the extent of maceration is determined after a defined period of time. The relative decrease of susceptibility can be determined in comparison with control explants.

As an alternative, eye cuttings infected with bacteria can be pricked out and cultivated in a greenhouse under moist or humid conditions. Lack of oxygen

results from silting of the earth, which supports the multiplication of the bacteria. The number of accumulated healthy sprouts is determined in comparison with the control explants. The extent of reduced susceptibility can be determined by this.

Throughout the specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement or any form of suggestion that the prior art forms part of the common general knowledge in Australia.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An expression system for anaerobic gene expression in higher plants, comprising the promoter GapC4 operably linked to a gene to be expressed, wherein the gene is a foreign gene.

2. The expression system according to claim 1, characterised by parts or variants of the promoter GapC4, wherein the inducibility under anaerobic conditions, of parts or variants of the promoter GapC4, is substantially comparable to that of wildtype promoter GapC4.

3. The expression system according to claims 1 and 2, wherein the gene encodes an antibacterial protein.

4. The expression system according to claim 3, wherein the gene encodes T4 lysozyme.

5. The expression system according to claims 1 and 2, wherein the gene is a resistance gene (r-determinants) which confers resistance against viruses, nematodes, bacteria and fungi.

6. The expression system according to claims 1 and 2, wherein the gene confers an insecticidal effect.

7. The expression system according to claims 1 and 2, wherein the gene is adapted to increase glycolysis.

8. The expression system according to claims 1 and 2, wherein the gene is adapted to increase fermentation.

9. The expression system according to claims 1 to 8 for use in all higher plants.

10. The expression system according to claims 1 to 8 for use in cultivated plants.

11. The expression system according to claim 10 for use in any one of potatoes, rice, grain, corn, tomatoes, brassicaceae, leguminous plants, cotton sugar beets or carrots.

12. A plant cell, containing an expression system according to any one of claims 1 to 11.

13. Transgenic higher plants, containing an expression system according to any one of claims 1 to 11.

14. Transgenic cultivated plants according to claim 13.

15. Transgenic potatoes containing an expression system comprising the promoter GapC4 operably linked to a gene encoding T4 lysozyme.

16. The seed of transgenic higher plant as claimed in claim 13, containing an expression system according to any one of claims 1 to 11.

17. The progeny of a transgenic higher plant as claimed in claim 13, containing an expression system according to any one of claims 1 to 11.

18. An expression system, a plant cell containing said expression system, a transgenic higher plant containing said expression system, seed of said transgenic higher plant or progeny of said transgenic higher plant substantially as herein described with reference to the examples.

DATED this 2nd day of August, 2000.

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